PTPN22 Is Genetically Associated with Risk of Generalized Vitiligo, but *CTLA4* Is Not

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Generalized vitiligo is an acquired, multifactorial, polygenic disease in which depigmented spots of skin, overlying hair, and mucus membranes result from autoimmune-mediated loss of melanocytes from affected areas. We examined single-nucleotide polymorphisms (SNPs) in the *PTPN22* and *CTLA4* genes in 126 Caucasian families with multiple cases of generalized vitiligo and associated autoimmune diseases, using a family-based association study design. The *PTPN22* 1858T allele of SNP rs2476601 is significantly associated both with generalized vitiligo and with an expanded autoimmunity phenotype. Individuals carrying the *PTPN22* 1858T allele had an allelic odds ratio (OR) of 2.16 for generalized vitiligo and a genotypic OR of 2.35 as C/T heterozygotes. Similarly, individuals carrying the *PTPN22* 1858T allele had an allelic OR of 2.05 for the expanded autoimmunity phenotype, and a genotypic OR of 2.19 for C/T heterozygotes. Examination of five SNPs in the *CTLA4* gene (rs1863800, rs231775, rs3087243, rs11571302, rs11571297, rs10932037) in the same 126 families yielded no evidence of allelic or genotypic association with either generalized vitiligo or the expanded autoimmune phenotype. These results implicate *PTPN22* in mediating susceptibility to generalized vitiligo and associated autoimmune diseases, but do not support a role for *CTLA4*.

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INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation of skin, overlying hair, and mucous membranes results from loss of melanocytes from the involved areas (reviewed in Hann and Nordlund, 2000; Nordlund *et al.*, 2006). Known for thousands of years because of its visually evident phenotype, vitiligo is the most common pigmentation disorder, affecting about 0.4% of Caucasians (Howitz *et al.*, 1977) and occurring with generally similar frequency in other populations (for example, Das *et al.*, 1985a; Lu *et al.*, 2007). Several different etiologic hypotheses have been suggested for generalized vitiligo (reviewed in Nordlund *et al.*, 2006), the most compelling of which involves a combination of unknown environmental and genetic factors interacting to contribute to autoimmune melanocyte destruction.

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Abbreviations: CI, confidence interval; LD, linkage disequilibrium; OR, odds ratio; SNP, single-nucleotide polymorphism

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Most cases of generalized vitiligo occur sporadically, although about 15–20% of patients report one or more affected first-degree relatives. Typically, familial aggregation of generalized vitiligo cases occurs in a non-Mendelian pattern that is suggestive of polygenic, multifactorial inheritance (Mehta *et al.*, 1973; Carnevale *et al.*, 1980; Hafez *et al.*, 1983; Das *et al.*, 1985a, b; Majumder *et al.*, 1988, 1993; Bhatia *et al.*, 1992; Nath *et al.*, 1994; Alkhateeb *et al.*, 2003; LaBerge *et al.*, 2005; Sun *et al.*, 2006), and formal genetic segregation analyses have indicated that multiple major loci contribute to vitiligo susceptibility in a complex interactive manner (Majumder *et al.*, 1993; Nath *et al.*, 1994; Sun *et al.*, 2006). Furthermore, patients with generalized vitiligo are also at increased risk of developing other autoimmune diseases, particularly autoimmune thyroid disease (Graves' disease and autoimmune hypothyroidism), rheumatoid arthritis, psoriasis, latent autoimmune diabetes of adults, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al.*, 2003; LaBerge *et al.*, 2005). These same diseases also occur at increased frequencies in vitiligo patients' first-degree relatives, suggesting that susceptibility to this group of autoimmune diseases is genetically determined.

Genetic linkage and association studies have implicated a number of different genes in susceptibility to generalized vitiligo (reviewed in Spritz, 2007), including several that are thought to play primary roles in the development of autoimmunity. These include the major histocompatibility complex (for example, Foley *et al.*, 1983; Finco *et al.*, 1991; Orecchia *et al.*, 1992; Ando *et al.*, 1993; Schallreuter *et al.*, 1993; al-Fouzan *et al.*, 1995; Zamani *et al.*, 2001; Arcos-

Burgos *et al.*, 2002; Tastan *et al.*, 2004; Fain *et al.*, 2006; Xia *et al.*, 2006; Liu *et al.*, 2007), *CTLA4* (Kemp *et al.*, 1999; Blomhoff *et al.*, 2005, Itirli *et al.*, 2005), and *PTPN22* (Canton *et al.*, 2005), and *NALP1* (Jin *et al.*, 2007a, b). Association of vitiligo with *PTPN22* and *CTLA4* is based on small case–control studies, a study design that is notoriously subject to both false-positive and false-negative errors due to population admixture and stratification (Hirschhorn *et al.*, 2002). To more rigorously test the association of vitiligo with variation in *PTPN22* and *CTLA4*, we carried out family-based association analyses of *CTLA4* and *PTPN22* single-nucleotide polymorphisms (SNPs) in 126 Caucasian families with multiple cases of generalized vitiligo, which included the same families in which we previously showed association to HLA markers (Fain *et al.*, 2006) and *NALP1* (Jin *et al.*, 2007a).

RESULTS

Family-based association analysis of *PTPN22*

We genotyped rs2476601 (*PTPN22* risk allele 1858T) in 712 individuals from 126 Caucasian extended families with multiple cases of vitiligo as well as other autoimmune diseases, derived from the United States and the United Kingdom (Alkhateeb *et al.*, 2003; LaBerge *et al.*, 2005; Jin *et al.*, 2007a). SNP rs2476601 was found to be in Hardy–Weinberg equilibrium in founders (individuals with no parents specified in the pedigree) in the 126 study families (Table 1).

As shown in Table 2, we found that the high-risk 1858T allele of rs2476601 was significantly associated ($P=0.0048$) with generalized vitiligo in the 126 study families. Genotypic analysis showed that the rs2476601 heterozygous C/T genotype was highly associated with vitiligo ($P=0.0012$). The *PTPN22* high-risk 1858T allele showed allelic association with generalized vitiligo [$P=0.024$, odds ratio (OR) 2.16 (95% confidence interval (CI) 1.22–3.82)] (Table 2) from conditional logistic regression analysis.

We also tested an expanded autoimmunity phenotype that included all of the autoimmune diseases with which vitiligo is epidemiologically associated (autoimmune thyroid disease, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, systemic lupus erythematosus, Addison's disease), considering any individual with either vitiligo or any other of these autoimmune diseases as ''affected''. As shown in Table 2, the rs2476601 1858T allele also showed allelic association ($P=0.0042$) with this expanded autoimmunity phenotype, which was confirmed by conditional logistic regression analysis [$P=0.03$, OR 2.05 (95% CI 1.19–3.53)]. Genotypic analysis, presented in Table 3, showed that the rs2476601 C/T genotype is significantly associated with the expanded autoimmunity phenotype ($P=0.003$), although association with the T/T genotype was not significant ($P=0.750$). Conditional logistic regression analysis provided very similar results, showing significant association of the C/T heterozygote with the autoimmune disease phenotype [$P=0.03$, OR 2.19 (95% CI 1.20–3.97)], but non-significant association of the T/T homozygote [$P=0.14$, OR 3.22 (95% CI 0.68–15.27)].

Table 1. Results of Hardy–Weinberg equilibrium (HWE) analysis of SNP genotypes among founders from 126 multiplex vitiligo-autoimmune disease families

SNP	Observed genotypes in founders	HWE *P*-value
PTPN22		
rs2476601	T/T 2 C/T 34 C/C 104	0.9170
CTLA4		
rs1863800	T/T 19 C/T 74 C/C 45	0.4210
rs231775	G/G 41 A/G 73 A/A 25	0.7492
rs3087243	A/A 23 A/G 76 G/G 40	0.4304
rs11571302	A/A 24 A/C 75 C/C 38	0.4602
rs11571297	G/G 29 A/G 75 A/A 34	0.5830
rs10932037	T/T 4 C/T 27 C/C 109	0.3750

SNP, single-nucleotide polymorphism.

Family-based association analysis of *CTLA4*

We also examined six SNPs in the *CTLA4* gene (rs1863800, rs231775, rs3087243, rs11571302, rs11571297, rs10932037) for association with generalized vitiligo and the expanded vitiligo-associated autoimmune phenotype in these families. None of the *CTLA4* SNPs tested exhibited either allelic (Table 2) or genotypic (Table 3) association with either generalized vitiligo or with the expanded autoimmunity phenotype. All but one of these SNPs clustered into a single linkage disequilibrium (LD) block (data not shown), and no multiple testing corrections were applied to the nominal *P*-value threshold ($P=0.05$) due to lack of any apparent association with disease.

DISCUSSION

We have carried out family-based association analyses of *PTPN22* and *CTLA4* SNPs in 126 Caucasian extended families with multiplex cases of vitiligo as well as other autoimmune diseases, confirming the association of generalized vitiligo with the functional *PTPN22* 1858T (R620W)-variant allele (OR 2.16, 95% CI 1.22–3.82) and the heterozygous C/T genotype (OR 2.35, 95% CI 1.25–4.43). Furthermore, we find that the *PTPN22* 1858T allele and C/T genotype are also associated with an expanded autoimmunity phenotype (allelic OR 2.05, 95% CI 1.19–3.53; genotypic OR 2.19, 95% CI 1.20–3.97) that includes generalized vitiligo or

Table 2. Allelic association analyses of *PTPN22* and *CTLA4* SNPs

SNP	FBAT[1] (*P*-value)	Regression on alleles (*P*-value)	Odds ratio (95% CI)
Vitiligo			
PTPN22			
rs2476601	0.0048[2]	0.024[2]	2.16 (1.22–3.82)
CTLA4			
rs1863800	0.3742	0.495	0.89 (0.63–1.24)
rs231775	0.5440	0.592	1.09 (0.79–1.52)
rs3087243	0.3518	0.683	0.934 (0.67–1.30)
rs11571302	0.3628	0.930	0.986 (0.71–1.36)
rs11571297	0.3942	0.858	1.03 (0.74–1.44)
rs10932037	0.8688	1.00	1.00 (0.61–1.65)
Expanded autoimmunity phenotype			
PTPN22			
rs2476601	0.0042[2]	0.03[2]	2.05 (1.19–3.53)
CTLA4			
rs1863800	0.2907	0.305	0.85 (0.62–1.16)
rs231775	0.4198	0.310	0.85 (0.62–1.16)
rs3087243	0.2460	0.370	0.87 (0.63–1.18)
rs11571302	0.2655	0.578	0.92 (0.67–1.25)
rs11571297	0.2920	0.465	1.13 (0.82–1.56)
rs10932037	0.8858	0.809	0.94 (0.59–1.52)

CI, confidence interval; FBAT, family-based association test; SNP, single-nucleotide polymorphism.
[1] *P*-values based on FBAT additive model.
[2] *P*-values corrected for three independent tests: two LD blocks in *CTLA4* and one SNP in *PTPN22*.

any of the other autoimmune diseases with which vitiligo is epidemiologically associated (Alkhateeb *et al.*, 2003; LaBerge *et al.*, 2005). These findings strongly support a causal role for the *PTPN22* 1858T allele of rs2476601 (or another variant with which 1858T is in close LD) in susceptibility to both generalized vitiligo and these other autoimmune diseases. We found no association of the *PTPN22* rs2476601 homozygous T/T genotype with either generalized vitiligo or the expanded autoimmune phenotype, most likely due to limited power resulting from the low frequency of the T allele (0.13 in this study) and the consequent small number of T/T homozygotes observed (8 of 712 individuals genotyped).

However, our analyses of the *CTLA4* SNPs showed no apparent allelic or genotypic association with either generalized vitiligo or the expanded autoimmunity phenotype. These results are in contrast with previous reports of genetic association of *CTLA4* markers with vitiligo based on small case–control studies (Kemp *et al.*, 1999; Blomhoff *et al.*, 2005; Itirli *et al.*, 2005). Kemp *et al.* (1999) reported the association of the 106-bp allele of a microsatellite polymorphism in the *CTLA4* 3′-untranslated region with vitiligo (especially autoimmune-associated) in 74 European Caucasian patients *versus* 173 controls. The same group (Blomhoff *et al.*, 2005) subsequently reported the association of *CTLA4* SNPs MH30 (23 kb 5′ of the *CTLA4* gene), rs3087243, rs11571302, and rs7565213 with vitiligo in 27 European Caucasian patients with associated autoimmune disease *versus* 140 controls; it is not clear whether the case and control cohorts in these two studies were independent. Itirli *et al.* (2005) reported the association of the 96-bp allele of the same *CTLA4* microsatellite in 36 Turkish patients *versus* 100 controls. It may be that these previous ''associations'' represent spurious false-positive results, due to either occult population stratification (reviewed in Hirschhorn *et al.*, 2002) or the very small sample size and highly unbalanced numbers of cases and controls in both studies. Furthermore, Blomhoff *et al.* (2005) did not correct for multiple testing, which would have rendered the reported *CTLA4* associations insignificant. Itirli *et al.* (2005) reported significance based on the observation of only four vitiligo patients *versus* two controls with the 96-bp allele, whereas the 112-bp allele, which showed the largest difference in allele frequency between cases and controls, was not significant, indicating that ''significance'' of the 96-bp allele almost certainly represents statistical fluctuation due to the very small number of observations.

PTPN22 and *CTLA4* are thought to function as general autoimmunity susceptibility loci (Brand *et al.*, 2005; Gregersen *et al.*, 2006). The *PTPN22* 1858T variant results in an arginine to tryptophan substitution that disrupts interaction between Lyp and Csk protein tyrosine kinases, dis-inhibiting T-cell activation (Siminovitch, 2004) and perhaps thereby increasing susceptibility to autoimmune disease (Brand *et al.*, 2005). The *PTPN22* 1858T variant has been associated with type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, Graves' disease (Bottini *et al.*, 2004; Siminovitch, 2004; Criswell *et al.*, 2005; Zhernakova *et al.*, 2005), and vitiligo (Canton *et al.*, 2005). In contrast, studies of patients with psoriasis and multiple sclerosis showed no association with the *PTPN22* risk allele in two family-based studies (Criswell *et al.*, 2005; Nistor *et al.*, 2005), suggesting that *PTPN22* may not truly be associated with these diseases.

Our study utilized a family-based design to test association of *PTPN22* and *CTLA4* with generalized vitiligo, an approach that is more rigorous than previous case–control studies of these genes in vitiligo. Our findings demonstrate that *PTPN22* can be included along with HLA and *NALP1* among those genes confirmed to play a role in polygenic susceptibility to generalized vitiligo, but do not support a role for *CTLA4*.

MATERIALS AND METHODS

Subjects

Peripheral blood or saliva samples were obtained from 712 individuals from 126 extended families with multiplex cases of generalized vitiligo as well as other autoimmune diseases, derived from the United States and United Kingdom. All families were of Caucasian origin (as self-reported) and had two or more family members with generalized vitiligo and at least one family member with one or more other autoimmune disease with which generalized vitiligo is epidemiologically associated (autoimmune thyroid disease, latent autoimmune diabetes in adults, psoriasis, pernicious anemia, systemic lupus erythematosus, rheumatoid arthritis,

Table 3. Genotypic association analyses of *PTPN22* and *CTLA4* SNPs

SNP	Genotype	FBAT[1] (*P*-value)	Regression on genotypes (*P*-value)	Odds ratio (95% CI)
Vitiligo				
PTPN22				
rs2476601	T/T	0.814	0.126	3.42 (0.71–16.54)
	C/T	0.0012[2]	0.024[2]	2.35 (1.25–4.43)
	C/C	0.999	Referent	
CTLA4				
rs1863800	T/T	0.807	0.542	0.797 (0.385–1.65)
	C/T	0.488	0.576	0.886 (0.579–1.35)
	C/C	0.327	Referent	
rs231775	A/A	0.408	0.592	0.830 (0.419–1.64)
	A/G	0.563	0.762	1.08 (0.657–1.77)
	G/G	0.944	Referent	
rs3087243	A/A	0.732	0.652	0.854 (0.430–1.70)
	A/G	0.552	0.922	0.978 (0.623–1.53)
	G/G	0.309	Referent	
rs11571302	A/A	0.448	0.752	0.892 (0.440–1.81)
	A/C	0.989	0.523	1.16 (0.738–1.82)
	C/C	0.528	Referent	
rs11571297	G/G	0.567	0.706	1.15 (0.553–2.40)
	A/G	0.957	0.325	1.36 (0.740–2.48)
	A/A	0.473	Referent	
rs10932037	T/T	0.848	1.00	1.00 (0.234–4.28)
	C/T	0.751	1.00	1.00 (0.572–1.75)
	C/C	0.799	Referent	
Expanded autoimmunity phenotype				
PTPN22				
rs2476601	T/T	0.750	0.140	3.22 (0.68 –15.27)
	C/T	0.003[2]	0.03[2]	2.19 (1.20 – 3.97)
	C/C	0.999	Referent	
CTLA4				
rs1863800	T/T	0.636	0.379	0.734 (0.368–1.46)
	C/T	0.553	0.358	0.829 (0.555–1.24)
	C/C	0.299	Referent	
rs231775	A/A	0.187	0.310	0.712 (0.369–1.37)
	A/G	0.271	0.933	1.02 (0.638–1.63)
	G/G	0.896	Referent	
rs3087243	A/A	0.557	0.372	0.748 (0.394–1.42)
	A/G	0.619	0.550	0.876 (0.568–1.35)
	G/G	0.256	Referent	
rs11571302	A/A	0.371	0.506	0.801 (0.416–1.54)
	A/C	0.992	0.955	1.01 (0.656–1.56)
	C/C	0.426	Referent	
rs11571297	G/G	0.454	0.418	1.32 (0.672–2.60)
	A/G	0.987	0.334	1.31 (0.759–2.24)
	A/A	0.403	Referent	
rs10932037	T/T	0.784	0.942	0.948 (0.223–4.02)
	C/T	0.965	0.789	0.930 (0.549–1.58)
	C/C	0.955	Referent	

CI, confidence interval; FBAT, family-based association test; SNP, single-nucleotide polymorphism.
[1]*P*-values based on FBAT genotype model.
[2]*P*-values corrected for 3 independent tests: 2 LD blocks in *CTLA4* and 1 SNP in *PTPN22*.

Addison's disease; Alkhateeb *et al.*, 2003; LaBerge *et al.*, 2005). Diagnostic criteria for generalized vitiligo were consistent with those of the Vitiligo European Task Force (Taïeb and Picardo, 2007). Exclusion criteria were atypical lesion distribution, congenital or static skin depigmentation (for example, birthmarks, piebaldism, Waardenburg syndrome), depigmentation secondary to the use of melanocytotoxic chemicals, inflammatory skin diseases (systemic lupus erythematosus, lichen planus, psoriasis), and post-infectious or post-traumatic causes.

All available affected and unaffected family members completed a clinical history questionnaire reporting vitiligo age of onset and course of treatment, and a checklist of approximately 50 autoimmune and autoinflammatory diseases. Each vitiligo patient completed a skin-lesion map. All data were reviewed by study investigators and staff, and individuals in whom diagnoses were uncertain based on standard diagnostic criteria (Nordlund *et al.*, 2006) were excluded from the study. This study conformed to the Declaration of Helsinki Principles and was approved by the Colorado Multiple Institutional Review Board and the South East Research Ethics Committee. Written, informed consent was provided by all study participants.

SNP genotyping

DNA was isolated from peripheral blood using a genomic DNA purification kit (Purgene, Gentra Systems) or from saliva obtained using the DNA self-collection kit (Oragene; DNA Genotek, Ottawa, Ontario, Canada). Genotyping was carried out for *PTPN22*-rs2476601 (1858C/T), and *CTLA4*-rs1863800, rs231775 (+49G), rs3087243 (CT60), rs11571302 (JO31), rs11571297 (JO27_1), rs10932037 (CTIC154_1) in 10-μl PCR reaction volumes with 20 ng genomic DNA using standard methods. SNP alleles were detected with the Applied Biosystems (ABI, Foster City, CA) PRISM SNaPshot Multiplex Kit using an ABI 3130 Genetic Analyzer. Automated genotyping was performed using ABI Genemapper version 3.7 software; all genotypes were manually checked for accuracy and allele calling consistency. Mendelian inheritance of all markers was checked through all levels of PedCheck (O'Connell and Weeks, 1998). Haplotype analysis of *CTLA4* SNPs was carried out using the error-screening routine of Merlin, version 1.0.0 (Abecasis *et al.*, 2002).

Statistical analyses

Deviations from expected Hardy–Weinberg proportions were tested for each SNP in founders (no parents in the pedigree) and in persons not in the lineage in all 126 families using χ^2-analysis, considering $P<0.05$ significant. Calculation of LD between *CTLA4* SNPs was performed using Haploview software (Barrett *et al.*, 2005), version 3.32, with haplotype blocks being determined by the method of Gabriel *et al.* (2002). Single-locus association analyses of each SNP were carried out using the family-based association test (Horvath *et al.*, 2001). Conditional logistic regression analysis was carried out using STATA, version 9.2, which uses family-based data to create a matched pseudo case–control data set, test for allelic or genotypic association and estimate an OR for disease risk (Cordell and Clayton, 2002). Nominally significant *P*-values (<0.05) were corrected for three independent tests based on LD analysis, using Haploview (two LD blocks defined in the *CTLA4* gene and one SNP in *PTPN22*).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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